Two-membered mixed cultures of methanogenic and aerobic bacteria in O₂-limited chemostats

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Three different co-cultures composed of a methanogenic and a strictly aerobic bacterium were grown under O₂-limitation in continuous cultures. The combinations used were (1) *Methanobacterium formicicum* with the aerobic heterotroph *Comamonas testosteroni*; (2) *M. formicicum* with a methanotrophic *Methylocystis* species; and (3) *Methanosarcina barkeri* with *C. testosteroni*. Although true steady-states were not obtained, growth and metabolic activity of the methanogenic and aerobic organisms occurred during O₂-limited growth of these mixed cultures over extended periods of time. Co-cultures with *C. testosteroni* were considerably more stable than those with *Methylocystis*. Co-cultures with *M. barkeri* were less O₂-sensitive than those with *M. formicicum*. *C. testosteroni* exhibited a higher O₂-affinity than *Methylocystis*, resulting in a lower dissolved oxygen tension and a superior protection of the methanogenic bacteria against O₂-poisoning than in mixed cultures with *Methylocystis*. The dissolved O₂-concentrations in the mixed cultures were below the detection limit of the O₂-probes used (0.2 µM). Calculations based on growth properties of pure cultures of *C. testosteroni*, *M. barkeri* and *M. formicicum* suggested that the dissolved O₂-concentrations in the mixed cultures, as well as the O₂-inhibition constants (apparent $K_i^{O_2}$) of the methanogens were in the nanomolar range.

Introduction

Methanogenic bacteria are extremely sensitive to O₂. They contain high concentrations of O₂-labile compounds and require a relatively low redox potential for growth (Mah & Smith, 1981; Morris, 1976; Vogels *et al.*, 1988). Although methanogens may possess some enzymic self-defence against O₂-toxicity, growth in pure cultures is completely blocked in the presence of O₂ (Jones *et al.*, 1983; Kengen *et al.*, 1991; Kiener *et al.*, 1988; Kiener & Leisinger, 1983; Kirby *et al.*, 1981; Patel *et al.*, 1984; Robertson & Wolfe, 1970; Smith & Hungate, 1958; Zehnder & Wuhrmann, 1977). Accordingly, the typical natural habitats of methanogens are environments containing relatively large anoxic areas, such as aquatic sediments, flooded soils, landfills, stratified waters and the intestinal tract of animals. In addition, they may abide in anoxic micro-niches established in predominantly oxic environments in nutrient-rich 'oases', such as detritus aggregates or faecal pellets (Alldredge & Cohen, 1987; Lloyd *et al.*, 1986; Oremland, 1988; Sieburth, 1987). In these habitats, methanogenic bacteria

are protected from O₂-damage by the high respiratory activity of aerobic (micro-) organisms.

Near oxic/anoxic interfaces, particularly in systems with very steep O₂-gradients, methanogenic bacteria can live very closely to strictly aerobic bacteria. Metabolic coupling of anaerobes and aerobes may occur through exchange of metabolites (Wimpenny, 1981). An illustrative example is the methane cycle in freshwater environments characterized by the production of methane by strictly anaerobic methanogenic bacteria within the anoxic zone of the sediment or the water column and the oxidation of methane by strictly aerobic methanotrophic bacteria located in a narrow zone at the oxic/anoxic interface where the diffusion profiles of methane and O₂ overlap (Rudd & Taylor, 1980).

Several methanogenic bacteria – *Methanococcus maripaludis*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum*, *Methanobacterium bryantii* and *Methanobrevibacter arboriphilus* – though metabolically inactive, have been shown to survive oxic periods quite well (Jones *et al.*, 1983; Kiener *et al.*, 1988; Kiener & Leisinger, 1983; Patel *et al.*, 1983; Robertson & Wolfe, 1970; Zehnder & Wuhrmann, 1977). Hence, it can be anticipated that in areas exposed to alternating oxic/anoxic conditions the microbial population can

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contain methanogens as well as strict aerobes, though their activity is separated in time, depending on the (temporal) presence or absence of O_2 .

In spite of their apparent mutually exclusive type of metabolism, there are some indications that methanogenic and aerobic bacteria may also share the same micro-habitat (growth without separation in time and/or in space). This paradox may be explained by the fact that under O_2 -limitation aerobic respiring micro-organisms can maintain such very low dissolved O_2 -concentrations that complete inhibition of methanogens is avoided (Scott *et al.*, 1985; Lloyd *et al.*, 1989; Gerritse *et al.*, 1990).

Co-existence of strictly anaerobic and aerobic bacteria is interesting because it facilitates interspecific microbial interactions including substrate competition, cross-feeding and co-operation during the degradation of organic matter. Defined laboratory cultures have been used successfully in revealing information on the co-existence of and interactions between such metabolically diverse organisms (Chapman *et al.*, 1992; Gerritse *et al.*, 1990, 1992; Gottschal & Szewzyk, 1985; Ohta *et al.*, 1990; Van der Hoeven & Gottschal, 1989; Wimpenny & Abdollahi, 1991). However, the anaerobic bacteria used thus far in mixed cultures with aerobes are *Desulfovibrio*, *Streptococcus*, *Clostridium* and *Veillonella* species, all of which are known to tolerate low concentrations of O_2 .

The aims of the present study were to investigate whether methanogenic bacteria, renowned for their very high sensitivity to O_2 , can also grow in defined mixed cultures with strict aerobes. In addition, such cultures may also provide quantitative information on the factors responsible for the very narrow range of O_2 -concentrations which may allow co-existence of such bacteria. To this end, growth of the methanogens *Methanobacterium formicicum* and *Methanosarcina barkeri*, and the aerobes *Comamonas testosteroni* and *Methylocystis* sp. was studied both in pure cultures and in mixed cultures in the presence of growth-limiting concentrations of O_2 .

Methods

Organisms. *Methanobacterium formicicum* strain Wolfe was isolated and identified at the Department of Microbiology of the University of Nijmegen, The Netherlands, and kindly supplied by Dr E. Raemakers-Franken. *Methanosarcina barkeri* DSM 800 is maintained in the strain collection of our laboratory. The methanotrophic bacterium *Methylocystis* sp. strain T-1 was obtained from Dr K. Takeda, who isolated this organism from a soil which leaked methane gas (Takeda, 1988). *Comamonas testosteroni* sp., was isolated in our laboratory and described previously (Gerritse *et al.*, 1990).

Media and growth conditions. The medium used was a modified version of that described by Zehnder and Wuhrmann (1977). Basal medium contained (values in $g\ l^{-1}$): KH_2PO_4 , 0.54; Na_2HPO_4 , 1.34; NH_4Cl , 0.3; $NaCl$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.04; $MgCl_2 \cdot 6H_2O$, 0.04;

Nitriloacetic acid (trisodium salt), 0.02; $FeCl_2 \cdot 4H_2O$, 0.01; $KAl(SO_4)_2$, 0.0001; $Na_2SeO_3 \cdot 5H_2O$, 0.00003; $Na_2WO_4 \cdot 2H_2O$, 0.00003; $ZnSO_4 \cdot 7H_2O$, 0.0001; $MnCl_2 \cdot 4H_2O$, 0.00003; H_3BO_3 , 0.0003; $CoCl_2 \cdot 6H_2O$, 0.0002; $CuCl_2 \cdot 2H_2O$, 0.00001; $NiCl_2 \cdot 6H_2O$, 0.00002; $Na_2MoO_4 \cdot 2H_2O$, 0.00003; resazurin, 0.001; yeast extract (BBL) 0.1; gelysate peptone (BBL) 0.1; and 1 $ml\ l^{-1}$ of a filter-sterilized (0.2 μm pore-size) vitamin solution according to Heijthuisen & Hansen (1986).

For oxidic batch cultures of *Methylocystis* and *C. testosteroni* the basal medium was supplemented with 0.2 g $Na_2SO_4\ l^{-1}$. Potassium-L-lactate (20 mM) was added as the carbon and energy source for *C. testosteroni*, whereas methanol (50–100 mM) or methane (50%, v/v, in the gas-phase) were provided for growth of *Methylocystis*.

Methanogenic bacteria were routinely maintained in anoxic batch cultures with medium amended with $NaHCO_3$ (30 mM) and volatile organic acids (1.2 mM-acetate, 0.4 mM-propionate, 0.2 mM-butyrate, 0.05 mM-2-methylbutyrate, 0.05 mM-isobutyrate, 0.05 mM-valerate and 0.05 mM-isovalerate). Anoxic media were prepared under a N_2/CO_2 (80:20, v/v) atmosphere in culture bottles or Hungate tubes sealed with butyl-rubber stoppers and reduced with 10 $ml\ l^{-1}$ of a (1.25%, w/v) $Na_2S \cdot 9H_2O$ /cysteine.HCl mixture. Sodium formate (100 mM) was supplied as the carbon and energy source for *M. formicicum* from a separately autoclaved stock solution. For growth of *M. barkeri*, the NaCl concentration of the medium was raised to 0.6 $g\ l^{-1}$ and methanol (100–150 mM) was added from an autoclaved (50%, v/v) stock solution.

Chemostat media were the same as those used for batch cultures of methanogenic bacteria with the exception that the sulphide/cysteine solution was replaced by 10 $ml\ l^{-1}$ of a thiosulphate/cysteine solution (pH 10) containing 5% (w/v) $Na_2S_2O_3 \cdot 5H_2O$ and 1.25% (w/v) cysteine.HCl. For chemostat cultures with *M. formicicum*, peptone was omitted and the yeast extract concentration was increased from 0.1 to 1 $g\ l^{-1}$. Formic acid was pumped from a concentrated solution into the culture vessel, resulting in a reservoir concentration (S_r) of 430–460 mM.

Chemostat culture vessels had a liquid volume of 400–500 ml and were equipped with sensors enabling continuous measurement and regulation of temperature, redox potential and pH. Stable measurement of dissolved O_2 with Ingold sensors over extended periods of time (several weeks) was not possible as these electrodes appeared to be poisoned in the reduced chemostat media. O_2 -concentrations in the gas phase were measured with a Servomex 1100 oxygen analyser. A description of the chemostat configuration was published previously (Gerritse *et al.*, 1990).

Chemostat cultures of *M. formicicum* were grown at 37 °C and pH 7.5, whereas for cultures with *M. barkeri* the temperature was 35 °C and the pH 6.9. The pH was maintained by automatic titration with 1 M-NaOH or 1 M-HCl. The chemostats were stirred by means of a magnetic stirrer and the head-space of the culture vessel was gassed with N_2/CO_2 gas (80:20, v/v), freed of traces of O_2 by passing over hot copper turnings. For O_2 -limited growth, air was mixed with the N_2/CO_2 flow-gas resulting in mixtures containing 0–2% (v/v) O_2 , which were supplied both via the head-space as well as via a submerged pipe into the culture liquid.

Cultures of *M. formicicum* had some tendency to adhere to the glass and electrodes present in the chemostat (< 10% of the total biomass in the culture). In cultures with *M. formicicum*, wall-growth was removed before co-culture experiments with aerobic bacteria were performed. Wall-growth was not observed in cultures of *M. barkeri*.

Cell quantification in mixed cultures. The biomass of the populations of individual species in mixed chemostat cultures was estimated by combining cell-carbon measurements and microscopic cell-counts. Cells of *M. barkeri* (large aggregated cocci), *M. formicicum* (long thin rods to filaments), *C. testosteroni* (thick straight rods) and *Methylocystis* (small oval rods) could readily be distinguished morphologically. The

ratio between cell-numbers of different species in mixed cultures was determined on a Carl Zeiss G42-110-e phase-contrast microscope in samples taken from continuous cultures, fixed with 0.4% formaldehyde. Numbers of bacterial cells per unit volume were obtained with a Bürker-Türk counting chamber. After appropriate dilution, 500–1500 cells were counted in at least duplicate samples. In aggregates of cells, individual cells were distinguished on the basis of a microscopically visible cell-wall. Subsequently, the amount of cell-carbon of the individual bacteria in the mixed cultures was calculated from the total cell-carbon concentration in the co-cultures and a cell-number/cell-carbon calibration determined in pure cultures. Cell counts in batch cultures of *M. formicicum*, *M. barkeri*, *C. testosteroni* and *Methylocystis* yielded values of 6.90×10^9 , 0.268×10^9 , 5.71×10^9 and 13.10×10^9 cells (mg cell-carbon) $^{-1}$, respectively.

Respirometry. O_2 -consumption rates were determined at 37 °C (*Methylocystis*) or 35 °C (*C. testosteroni*) in a Yellow-Springs-type biological oxygen monitor equipped with a polarographic oxygen electrode connected to a Keithley model 485 picoamperometer for increased sensitivity at very low O_2 -concentrations (Gerritse *et al.*, 1992). This method allowed detection of O_2 at concentrations $> 0.01 \mu M$. O_2 -consumption of *C. testosteroni* was analysed with dilute resting cell suspensions in medium containing chloramphenicol and 20 mM-L-lactate. To anoxic cell-suspensions of *C. testosteroni*, O_2 was added to a concentration of about $10 \mu M$ by injection of air-saturated water. O_2 -utilization by *Methylocystis* was analysed with resting cell suspensions saturated with an air/methane mixture (about 50%, v/v) or in air-saturated suspensions containing 50 mM-methanol. Kinetic constants, apparent K_m , O_2 and $Q_{O_2}^{max}$ values, were obtained from direct linear plots. It was assumed that air-saturated suspensions contained $218 \mu M$ dissolved O_2 .

Determination of growth kinetics. Maximum specific growth rates (μ_{max} values) were determined by following the change in optical density at 660 nm (OD_{660}) in batch cultures, or in continuous cultures with the dilution (D) rate 20–50% above the μ_{max} (wash-out experiments). Half-saturation constants for growth (K_s) were obtained from specific growth rates and residual formate or methanol concentrations ($\bar{s}$) in steady-state cultures of *M. formicicum* or *M. barkeri*, respectively. It was assumed that growth of these bacteria followed Monod-type kinetics and the corresponding K_s values were estimated from direct linear plots.

Analytical procedures. Quantification of gases (CH_4 , CO_2 and O_2), total organic carbon and cell-carbon was done as described previously (Gerritse *et al.*, 1990). Formate concentrations were determined colorimetrically according to the method of Lang & Lang (1972). L-Lactate concentrations were determined gas-chromatographically as described by Nanninga & Gottschal (1985). Methanol was measured on a Packard 427 gas-chromatograph equipped with a Porapack R column (80/100 mesh) kept at a temperature of 114 °C. The temperature of the injector and the (flame-ionization) detector was 120 °C.

Chemicals. All chemicals used were of analytical grade quality and obtained from commercial companies, except L-lactic acid (Fluka), which contained 90% L-lactic acid in water.

Results

Growth and O_2 -uptake kinetics of pure cultures of *Comamonas testosteroni* and *Methylocystis* sp.

Maximum specific-consumption rates ($Q_{O_2}^{max}$) and half-saturation constants (apparent K_m) for O_2 were obtained

respirometrically with cell-suspensions of *Methylocystis* and *C. testosteroni*, grown separately in oxic batch cultures.

The maximum specific growth rate (μ_{max}) of *C. testosteroni* in a batch culture at 35 °C on 20 mM-L-lactate was $0.39 (\pm 0.02) h^{-1}$. Rates of O_2 -consumption by resting cell suspensions of *C. testosteroni* revealed a $Q_{O_2}^{max}$ of $22.4 \mu mol (mg \text{ cell-carbon})^{-1} h^{-1}$ and an apparent K_m of $0.45 \mu M$ (Table 1). Cell suspensions of *C. testosteroni* did not oxidize methane or methanol.

In a shake culture (150 r.p.m.) at 37 °C with an air/methane mixture (50:50, v/v) in the head-space, *Methylocystis* grew with a μ_{max} of $0.09 h^{-1}$. When the carbon and energy source of an exponential-phase culture of *Methylocystis* was switched from methane to methanol (100% air in the head-space and addition of 60 mM-methanol), exponential growth continued without a lag at a rate of $0.08 h^{-1}$. Methane-grown cells of *Methylocystis* oxidized methane as well as methanol. The affinity for O_2 was considerably higher with methanol than with methane as the substrate (Table 1).

Growth of *Methanobacterium formicicum* and *Methanosarcina barkeri* in anoxic continuous cultures

Some growth parameters of *M. formicicum* and *M. barkeri* were determined in continuous cultures with formate or methanol, respectively, as the growth substrates.

Determination of μ_{max} through wash-out experiments using anoxic steady-state cultures of *M. formicicum* ($D = 0.026 h^{-1}$, pH 7.5, temperature 37 °C) revealed relatively low values. Cell-yield values and the K_s for formate were also measured in this steady-state culture (Table 2). In batch culture on 100 mM-formate similar growth rate ($\mu_{max} = 0.028 h^{-1}$) and yield data ($0.15 g \text{ mol}^{-1}$) were obtained.

With *M. barkeri*, methanol-limited steady-state cultures ($D = 0.026 h^{-1}$, pH 6.9, temperature 35 °C) were obtained (Table 2). At a dilution rate of $0.05 h^{-1}$ *M. barkeri* washed-out at a rate which indicated a μ_{max} of $0.042 h^{-1}$, very close to that found in batch culture ($0.045 h^{-1}$).

The stoichiometries of methane production observed in the present study (Table 2) agreed well with the known equations for the methanogenic conversion of formate and methanol reported previously (Vogels *et al.*, 1988).

Washed cell-suspensions from chemostat cultures of *M. formicicum* or *M. barkeri* did not consume measur-

Table 1. Kinetic parameters for O_2 -consumption by resting cells of *Comamonas testosteroni* and *Methylocystis* sp. pre-grown on L-lactate or methane, respectively

Values are means of at least three determinations $\pm$ standard deviation.

Species	Substrate oxidized	$Q_{O_2}^{\max}$ ($\mu\text{mol mg}^{-1} \text{h}^{-1}$)	Apparent $K_m^{O_2}$ (μM)	$Q_{O_2}^{\max}/K_m^{O_2}$
<i>C. testosteroni</i>	L-Lactate	22.4 ± 4.7	0.45 ± 0.14	49.8
<i>Methylocystis</i>	Methane	7.4 ± 0.2	4.7 ± 0.75	1.6
	Methanol	18.6 ± 0.3	1.9 ± 0.37	9.8

Table 2. Growth parameters obtained from steady-state cultures of *Methanobacterium formicicum* and *Methanosarcina barkeri*

The dilution rate of the steady-state cultures was 0.026 h^{-1} .

Species	Substrate (mm in reservoir)	$Q_{\text{substrate}}$ ($\text{mmol g}^{-1} \text{h}^{-1}$)	Q_{CH_4}	$Y_{\text{substrate}}$ (g mol^{-1})	$\bar{S}_{\text{substrate}}$ (mm)	$\mu_{\max}^*$ (h^{-1})	K_s (mm)
<i>M. formicicum</i>	Formate (460)	142.4	40.1	0.18	9.1	0.034	2.8
<i>M. barkeri</i>	Methanol (115)	10.6	6.94	2.44	0.3	0.042	0.18

* Determined from the rate of wash-out at a dilution rate of 0.05 h^{-1} .

able amounts of O_2 [$< 0.04 \mu\text{mol} (\text{mg cell-carbon})^{-1} \text{h}^{-1}$] in the presence of either formate or methanol.

O₂-limited mixed cultures with Methanobacterium formicicum

(1) *Co-culture of M. formicicum and C. testosteroni*. An anoxic formate-limited chemostat culture of *M. formicicum* ($D = 0.026 \text{ h}^{-1}$) was inoculated with about 150 ml of a L-lactate-grown batch culture of *C. testosteroni* (Fig. 1a). From $t = 0$, the culture liquid was supplied with a flow of N_2/CO_2 gas containing O_2 to a concentration of 0.18% (v/v). This gas mixture was supplied at a rate of about 2.5 l h^{-1} ($450 \mu\text{M-O}_2 \text{ h}^{-1}$). O_2 was consumed at a rate of $138 \pm 19 \mu\text{M h}^{-1}$ during this initial period of O_2 -limited growth. Residual L-lactate remained $> 17 \text{ mM}$ and the redox-reading and dissolved O_2 below -175 mV and $0.2 \mu\text{M}$, respectively.

A sharp decrease in the rate of methane formation occurred within the first 5 h of the experiment, probably caused by dilution of the population of *M. formicicum* present in the chemostat (Fig. 1c). During the next 2.5 volume changes (65 h), methane formation was restored partially but did not reach the initial anoxic value. Both *M. formicicum* and *C. testosteroni* washed-out, though at a lower rate than the rate of dilution. Even a decrease in

the dilution rate (from 0.026 to 0.020 h^{-1}) at $t = 71$ did not prevent a gradual decrease in methane formation.

After O_2 -limited growth for 165 h (5.3 volume changes) the medium supply was stopped but the introduction of O_2 into the culture was maintained at $450 \mu\text{M h}^{-1}$. The populations of *M. formicicum* and *C. testosteroni* both increased at the expense of the residual formate and L-lactate, respectively, present in the culture vessel, indicating simultaneous growth of both organisms.

(2) *Co-culture of M. formicicum and Methylocystis* sp. The aerobic methanotrophic bacterium *Methylocystis* sp. strain T1 was pre-grown on methane in a batch culture and inoculated into an anoxic formate-limited chemostat ($D = 0.028 \text{ h}^{-1}$, $S_{\text{formate}} = 430 \text{ mM}$) of *M. formicicum*. Although *Methylocystis* should be able to grow at the expense of methane produced by *M. formicicum*, the poor affinity for O_2 would preclude significant growth on methane (see above) at very low O_2 -concentrations. Therefore some methanol (initial concentration 57 mM) was added to the culture vessel to support O_2 -limited growth of this bacterium. Gradual introduction of air into the flow-gas resulted in an oxygen supply rate of $400\text{--}500 \mu\text{M h}^{-1}$ and a rate of O_2 -consumption of $30\text{--}50 \mu\text{M h}^{-1}$. Nevertheless, wash-out of the population of *Methylocystis* and methanol occurred at a rate equal to the dilution rate, indicating

Fig. 1. Time-course of a mixed continuous culture of *Methanobacterium formicicum* and *C. testosteroni*. *C. testosteroni* was inoculated into a formate-limited chemostat culture ($D = 0.026 \text{ h}^{-1}$, pH 7.5, temperature 37°C) of *M. formicicum* at $t = 0$. Also at $t = 0$, the resulting co-culture containing 57 mg *M. formicicum* cell-carbon l⁻¹ and 32 mg *C. testosteroni* cell-carbon l⁻¹ was exposed to limiting amounts of O₂ (0.18%, v/v, in the gas-flow). At $t = 71$, D was reduced from 0.026 h^{-1} to 0.020 h^{-1} and at $t = 165$ the medium supply was stopped. Carbon and energy sources in the reservoir medium were formate ($S_f = 460 \text{ mM}$) for *M. formicicum* and L-lactate ($S_L = 20 \text{ mM}$) for *C. testosteroni*. (a) Biomass of the population of *M. formicicum* (○) and *C. testosteroni* (●); —, rate of wash-out in the absence of growth. (b) Residual formate (●); —, formate accumulation in the absence of consumption. (c) Rate of methane formation (●).

that this bacterium was not able to grow at the expense of methanol (or methane) oxidation under these severely O₂-limited conditions.

Growth of *M. formicicum* was also dramatically inhibited in this co-culture with *Methylocystis*. Under O₂-limitation, methane formation was reduced by more than 70% and accumulation of formate and wash-out of the methanogen occurred at a rate nearly equal to the

dilution rate. After 3 volume changes, the redox-reading had increased from about -200 to -80 mV . When strictly anoxic conditions were restored at that moment, wash-out of *Methylocystis* continued, but growth of *M. formicicum*, formate consumption and methane production were resumed instantaneously.

O₂-limited mixed cultures of Methanosarcina barkeri and C. testosteroni

In a preliminary experiment (not shown), a batch culture of *C. testosteroni* was introduced into an anoxic methanol-limited steady-state culture of *M. barkeri* ($D = 0.026 \text{ h}^{-1}$, $S_{\text{methanol}} = 115 \text{ mM}$, $S_{\text{L-lactate}} = 21 \text{ mM}$). O₂ (0.7%, v/v) was included in the N₂/CO₂ gas and supplied at a rate of $260\text{--}280 \mu\text{M h}^{-1}$. Under these conditions, the mixed culture consumed O₂ at a rate of $11\text{--}13 \mu\text{M h}^{-1}$. After 4.4 volume changes (170 h) following inoculation, the density of *C. testosteroni* had dropped from 19 to 4 mg cell-carbon l⁻¹. However, the density of *M. barkeri* ($248\text{--}251 \text{ mg cell-carbon l}^{-1}$), the residual methanol concentration ($0.3\text{--}0.4 \text{ mM}$) and the rate of methane production ($1.85\text{--}1.95 \mu\text{M h}^{-1}$) had not changed significantly within this period, indicating that *M. barkeri* was capable of growth and methane formation under these O₂-limited conditions, but that the density of *C. testosteroni* dropped to unacceptably low levels.

The results of a very similar experiment, though with a somewhat higher O₂-supply (2%, v/v, at the same flow-rate) to stimulate growth of *C. testosteroni* are shown in Fig. 2. The biomass of *M. barkeri* in this mixed culture remained about 10% lower than in the monoculture. However, the time-course of the change in cell-carbon of *M. barkeri* (Fig. 2a) clearly showed that after the shift to O₂-limited conditions the methanogen grew with a specific growth rate corresponding to the dilution rate of the culture (0.026 h^{-1}) (Fig. 2d). Yet, methanol consumption appeared slightly less than its supply, resulting in a slow increase in the residual methanol concentration (Fig. 2b).

When the dilution rate of the mixed culture was raised from 0.026 h^{-1} to 0.039 h^{-1} at $t = 142 \text{ h}$, the population of the methanogen washed-out gradually (Fig. 2a). Nevertheless the specific rates of growth (μ) and methane production (Q_{CH_4}), expressed per unit of biomass of *M. barkeri*, increased after this shift to a higher dilution rate.

In these cultures, *C. testosteroni* maintained a population density of about 10 mg l^{-1} at a dilution rate of 0.026 h^{-1} and showed a drop to about 6 mg l^{-1} at $D = 0.039 \text{ h}^{-1}$ (Fig. 2a). Residual L-lactate (not shown) remained at a saturating concentration for *C. testosteroni* ($> 17 \text{ mM}$) as a consequence of the limited supply of O₂. In the mixed cultures of *C. testosteroni* and *M. barkeri*, the redox reading always remained between -150 and

Fig. 2. Time-course of a mixed continuous culture of *Methanosarcina barkeri* and *C. testosteroni*. *C. testosteroni* was inoculated into a methanol-limited chemostat culture ($D = 0.026 \text{ h}^{-1}$, pH 6.9, temperature 35°C) of *M. barkeri* at $t = 0$. The co-culture containing $260 \text{ mg } M. barkeri \text{ cell-carbon l}^{-1}$ and $17 \text{ mg } C. testosteroni \text{ cell-carbon l}^{-1}$ was subsequently exposed to limiting amounts of O_2 (2%, v/v, in the gas-flow). At $t = 142$, D was increased from 0.026 h^{-1} to 0.039 h^{-1} . Carbon and energy sources available in the reservoir medium were methanol ($S_r = 115 \text{ mM}$) for *M. barkeri* and L-lactate ($S_r = 21 \text{ mM}$) for *C. testosteroni*. (a) Biomass of the population of *M. barkeri* (○) and *C. testosteroni* (●); ———, rate of wash-out in the absence of growth. (b) Residual methanol (●); ———, methanol accumulation in the absence of consumption. (c) Rate of methane formation (○) and O_2 -consumption (●). (d) Specific growth-rate of the population of *M. barkeri* (---), *C. testosteroni* (—) and the dilution rate (---) of the mixed culture.

—250 mV, and the dissolved O_2 -concentration remained below the detection limit of the O_2 -probe ($< 0.2 \mu\text{M}$).

Discussion

The results presented in this paper show that methanogenic bacteria can be grown in mixed cultures with aerobic bacteria in chemostats, under O_2 -limiting conditions. Similar results were obtained earlier with co-cultures composed of strictly aerobic and anaerobic fermentative or sulphate-reducing bacteria (Chapman *et al.*, 1992; Gerritse *et al.*, 1990, 1992; Gottschal & Szewzyk, 1985; Ohta *et al.*, 1990; Van der Hoeven & Gottschal, 1989; Wimpenny & Abdollahi, 1991).

Most of the growth parameters (K_s , $\mu_{\max}$ and cell yield) obtained in continuous cultures of *Methanobacterium formicicum* and *Methanosarcina barkeri* with formate or methanol as the limiting substrates, respectively, were similar to those reported in other studies (Chua & Robinson, 1983; Schauer & Ferry, 1980; Schauer *et al.*, 1982; Weimer & Zeikus, 1978).

Real steady-states of mixed cultures of the methanogenic and aerobic bacteria were not obtained. Without exception the introduction of small amounts of O_2 led to a decrease in the rate of methane and biomass formation,

together with a rise in the residual concentration of the carbon and energy source of the methanogen. Nevertheless, metabolism and growth of *M. formicicum* as well as that of *M. barkeri* were evident in all the mixed cultures tested. The mixed culture of *M. barkeri* and *C. testosteroni* came closest to a true steady-state, followed by *M. formicicum* plus *C. testosteroni* and the combination of *M. formicicum* plus *Methylocystis* doing less well.

The observation that metabolism of *M. formicicum* was more inhibited in a mixed culture with *Methylocystis* than with *C. testosteroni* is most likely explained by the considerably (5 to 30 times) higher apparent O_2 -affinity of the latter organism. Particularly with methane as the substrate, the O_2 -consumption capacity of *Methylocystis* was relatively low. This suggests that methane monooxygenase, catalysing the initial oxidative step in methane metabolism by methanotrophic bacteria (the oxidation of methane to methanol) limits the rate of O_2 -utilization by *Methylocystis*. Similar observations were reported for *Methylosinus trichosporium* (Best & Higgins, 1981; Joergensen, 1985).

Because mixed cultures of *C. testosteroni* and *M. barkeri* were relatively stable and easy to grow, they were analysed in more detail. Although *M. barkeri* could be

maintained in a seemingly stable O₂-limited mixed culture with *C. testosteroni* for more than 8 volume changes (> 8 d), this did not represent truly stable co-existence of the methanogen and the aerobe. Apparently, the O₂-affinity of *C. testosteroni* is slightly insufficient to establish a dissolved O₂-concentration low enough to allow growth of the methanogen at a rate equal or above the dilution rate of the chemostat.

A major obstacle for the quantification of the inhibitory effect of O₂ on the growth of *M. formicicum* and *M. barkeri* is that suppression of their growth-rate took place at O₂-concentrations not detectable with the O₂-probes in the cultures. The same problem was encountered by Scott *et al.* (1985) and Lloyd *et al.* (1989) in determining the effect of O₂ on methanogenesis. In well-mixed samples of rumen liquid, anoxic digesters or suspensions of *M. barkeri*, exposed to O₂, methanogenesis was consistently inhibited before dissolved O₂ became detectable even with a Photobacterium-probe or a membrane-inlet mass spectrometer (detection limits of 30 and 250 nM, respectively).

An estimate of the K_i^{O₂}, the O₂-concentration repressing the specific growth rate of the methanogenic bacteria to $\frac{1}{2}\mu_{\max}$, may be obtained indirectly from our results of the mixed cultures with *C. testosteroni*. It is reasonable to assume that under O₂-limiting conditions, with saturating concentrations of a carbon and energy source and other nutrients, the specific growth rate (μ) of a strictly aerobic bacterium depends on the dissolved O₂ concentration ([O₂]) according to the Monod equation (Owens & Legan, 1987).

$$\mu = \mu_{\max} \cdot [\text{O}_2] / (K_s + [\text{O}_2]) \quad (3)$$

By further assuming that the half-saturation constant (K_s) for O₂-limited growth of *C. testosteroni* is similar to the apparent K_m for O₂-consumption determined respirometrically, it follows that the dissolved O₂-concentrations in the co-culture of *M. barkeri* and *C. testosteroni* can be assessed as follows.

$$[\text{O}_2] = \mu \cdot K_m / (\mu_{\max} - \mu) \quad (4)$$

In this equation, μ represents the specific growth rate of *C. testosteroni* in the mixed culture, $\mu_{\max}$ is the maximum specific growth rate on L-lactate at saturating O₂ and lactate concentrations (0.39 h⁻¹), and K_m is the half-saturation constant for O₂-consumption obtained respirometrically (0.45 μM) with *C. testosteroni* grown at 35 °C in a batch culture on L-lactate. This latter value is close to the apparent K_m (0.35 μM) determined previously for this bacterium grown in pure culture on L-lactate in an O₂-limited chemostat culture (*D* = 0.1 h⁻¹) at 30 °C (Gerritse *et al.*, 1992).

The dissolved O₂-concentrations in the O₂-limited co-cultures of *C. testosteroni* and *M. barkeri* thus calculated

were in the range 6 to 40 nM using the values for μ shown in Fig. 2(d). In these cultures, the specific growth rate of *M. barkeri* varied from 0.022 to 0.032 h⁻¹, which is within 50–75 % of the anaerobically determined $\mu_{\max}$ on methanol (0.042 h⁻¹). Consequently, the apparent K_i^{O₂} of *M. barkeri* is presumably slightly higher than 40 nM (about 50–60 nM). Similar calculations for the mixed culture of *C. testosteroni* with *M. formicicum* suggest that this latter methanogen is less tolerant to O₂ than *M. barkeri*, with a K_i^{O₂} of about 10–20 nM. This difference in O₂-sensitivity could be based on the ability of *M. barkeri* to form cell-clusters which may help to minimize the exposure to O₂ of cells present in the interior of such cell-aggregates (Kiener & Leisinger, 1983). In our mixed-culture experiments, cell-aggregates of *M. barkeri* were composed of about 5–50 cells. Obviously, since these inhibition constants are estimates obtained in an indirect way, they should be interpreted cautiously and regarded as maximum values.

It is of interest to note that these experiments clearly indicate that the level of O₂-tolerance of the methanogenic bacteria used in this study is in the same order of magnitude as the dissolved O₂-concentrations maintained under O₂-limitation by *C. testosteroni*. The fact that such metabolically diverse organisms as strict aerobes and methanogens can thrive simultaneously in the same habitat strongly suggests the possibility of a very direct coupling of the metabolic capacities of these bacteria. This not only influences our views on the flow of carbon substrates in natural low-O₂ environments but it may also prove particularly useful for the development of biological purification systems (Gerritse *et al.*, 1992).

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